Fig. 8d. Parasite Density on D28

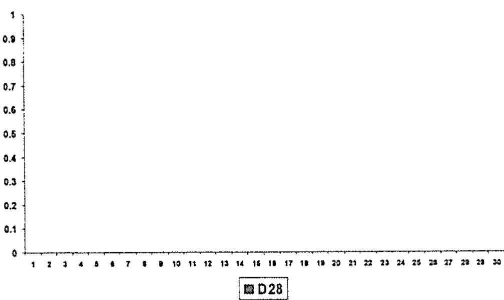

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Fig. 8e. Density of The Parasites

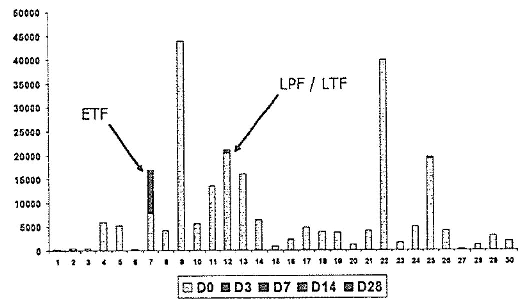

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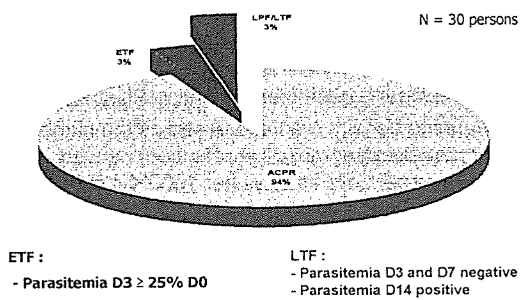
Fig. 9. Clinical Response



ETF :
- Parasitemia D3 ≥ 25% D0

LTF :
- Parasitemia D3 and D7 negative
- Parasitemia D14 positive

27

Conclusions

- There were vivax malaria in Tanjung Pura subdistrict
- Clinical Response :
  - Approximately 94% Adequate Clinical and Parasitological Response
  - 3% --- Early Treatment Failure
  - 3% --- Late Treatment Failure
  - Total Treatment Failure (ETF + LTF) < 25% from 30 cases
- Tanjung Pura subdistrict was :

  "The Resistance-available area"

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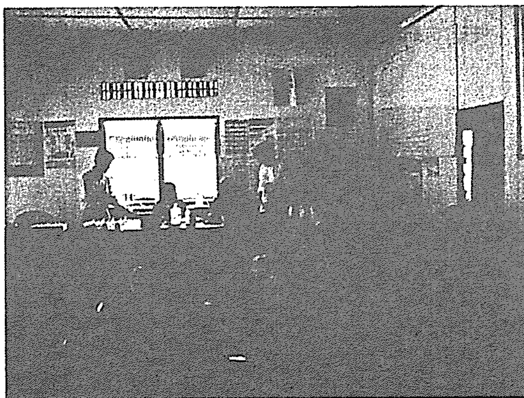

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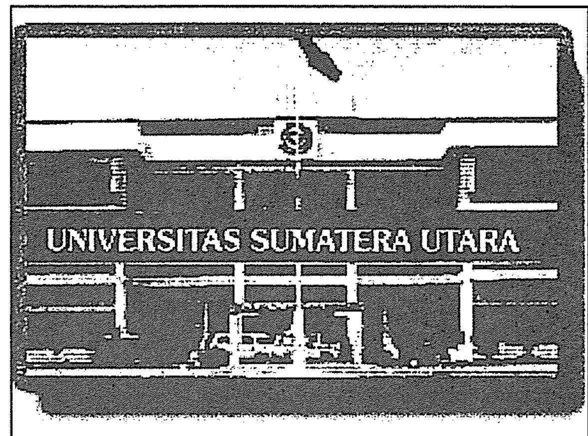

Diagnosis was confirmed by

- **Clinical assessment**
  Examining all patients enrollment to asses vital sign, blood pressure, heart rate and temperature
- **Laboratory Investigation**
  - Cappilary blood was drawn by finger prick, thick and thin blood smears examination in Giemsa-stained
  - Patients were followed up at D3, D7, D14, D28 and whenever sign of emergency occasionally occurs

33

Laboratory Investigation

- Thick smear – useful for detecting malaria
- Thin smear – useful for speciating Plasmodia
- Parasite density was expressed as the number of parasites per micro-liter of blood, derived from the numbers of parasites per 1000 rbc in a thin smear stained with giemsa or calculated from the wbc in thick smear

34

Fig. 4. Plasmodium vivax (thin blood smears)

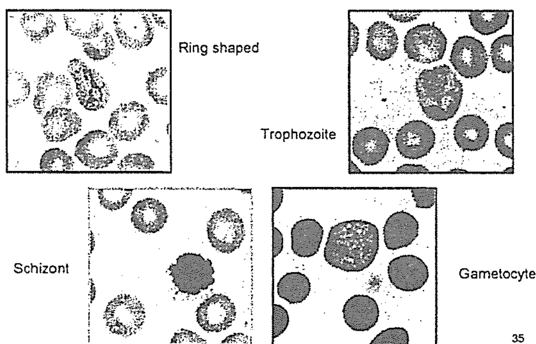

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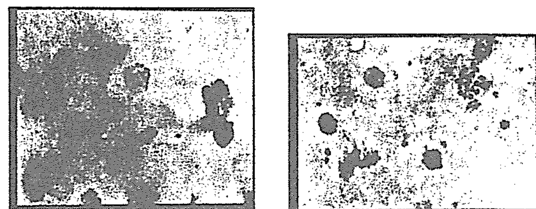
Fig. 5. Plasmodium vivax (thick blood smears)



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Chloroquine

- Tablet containing : 150 mg of base/tablet
- Dose :
  - Day 1: 10 mg/kg bw/day
  - Day 2: 10 mg/kg bw/day
  - Day 3: 5 mg/kg bw/day

Primaquine

- Tablet containing : 7.5 mg of base/tablet
- Dose : 0.25 mg/kg bw/day for 14 days

37

Early Treatment Failure (ETF) (WHO,2003)

- Development of danger signs or severe malaria on Day 1, Day 2 or Day 3, in the presence of parasitemia
- Parasitaemia on Day 2 higher than Day 0 count, irrespective of axillary temperature
- Parasitemia on Day 3 with axillary temperature 37.5 ° C
- Parasitemia on Day 3 ≥ 25 % of count on Day 0.

38

Late Clinical Failure (LCF) (WHO,2003)

- Development of danger signs or severe malaria after Day 3 in the presence of parasitemia, without previously meeting any of the criteria of early treatment failure
- Presence of parasitemia and axillary temperature 37.5 ° C (or history of fever) on any day from Day 4 to Day 28, without previously meeting any of the criteria of early treatment failure

39

Late Parasitological Failure (LPF) (WHO,2003)

- Presence of parasitemia on any day from Day 7 to Day 28 and axillary temperature < 37.5 ° C, without previously meeting any of the criteria of early treatment failure or late clinical failure

40

Adequate Clinical and Parasitological Response (ACPR)

(WHO,2003)

- Absence of parasitemia on Day 28 irrespective of axillary temperature without previously meeting any of the criteria of early treatment failure or late clinical failure or late parasitological failure

41

Discussion

- Percentage of vivax malaria in female is higher than male, but we suspect that the result came from the fact that on the day when the samples were taken, many men went out to the sea for fishing
- The ETF patient was still observed until D14, because no dangerous sign nor symptons of severe malaria was found during the period → Medications was admitted on day 14
- All the Treatment Failure patients responded to the alternative treatment

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Discussion

- Molecular genotyping using PCR technology should be used to distinguish recrudescent parasites from newly acquired infections
- Early diagnosis and treatment of existing cases is fundamental to breaking the human-mosquito-human cycle
- Providing more information to society about malaria especially the consequence of irrasional antimalarial intake

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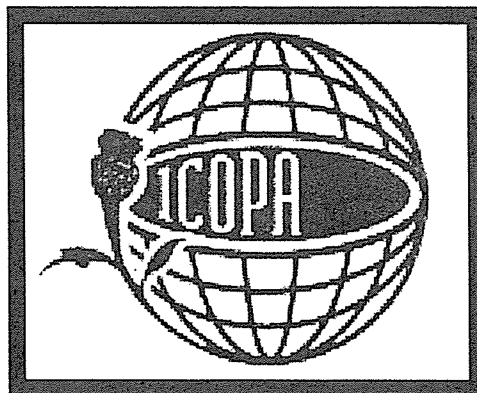

MEDIMOND

INTERNATIONAL PROCEEDINGS

Genetical and Biological Characteristics of Gastric *Cryptosporidium* spp.

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Summary

We investigated gastric types of *Cryptosporidium*. We isolated *C. muris* type oocysts from beef cattle in Miyagi, Japan, and the type was a novel type because of the difference of the infectivity to mice compared to that isolated in the U.S. We denoted it as *C. andersoni* Kawatabi type. It parasitized in the abomasum of cattle and the stomach of SCID mice, and had a relatively low infectivity to cattle. We also isolated *Cryptosporidium* from large Japanese field mice that parasitized to the stomach. The infectivity of the isolate to wild and laboratory mice was slightly different from that of *C. muris*. Phylogenetic analysis of DNA sequence of the 18S rRNA indicated that the isolate was a member of the *C. muris* cluster. This isolate was denoted as *Cryptosporidium muris* Japanese field mouse genotype as a novel genotype of *C. muris*.

Introduction

Cryptosporidium species are protozoan parasites that infect gastrointestinal epithelial cells of a wide range vertebrates, including humans. Gastric *Cryptosporidium* spp. have not been well analyzed, compared to intestinal *Cryptosporidium* spp., e.g. *C. parvum* and *C. hominis*. Then, we compared morphological, biological and genetical characteristics of gastric types of *Cryptosporidium* spp. that were isolated from rats, cattle, and wild mice.

Materials and Methods

Fecal samples were collected from grazing cattle and wild mice on a farm in Miyagi Prefecture in the northern part of the main island of Japan [5].

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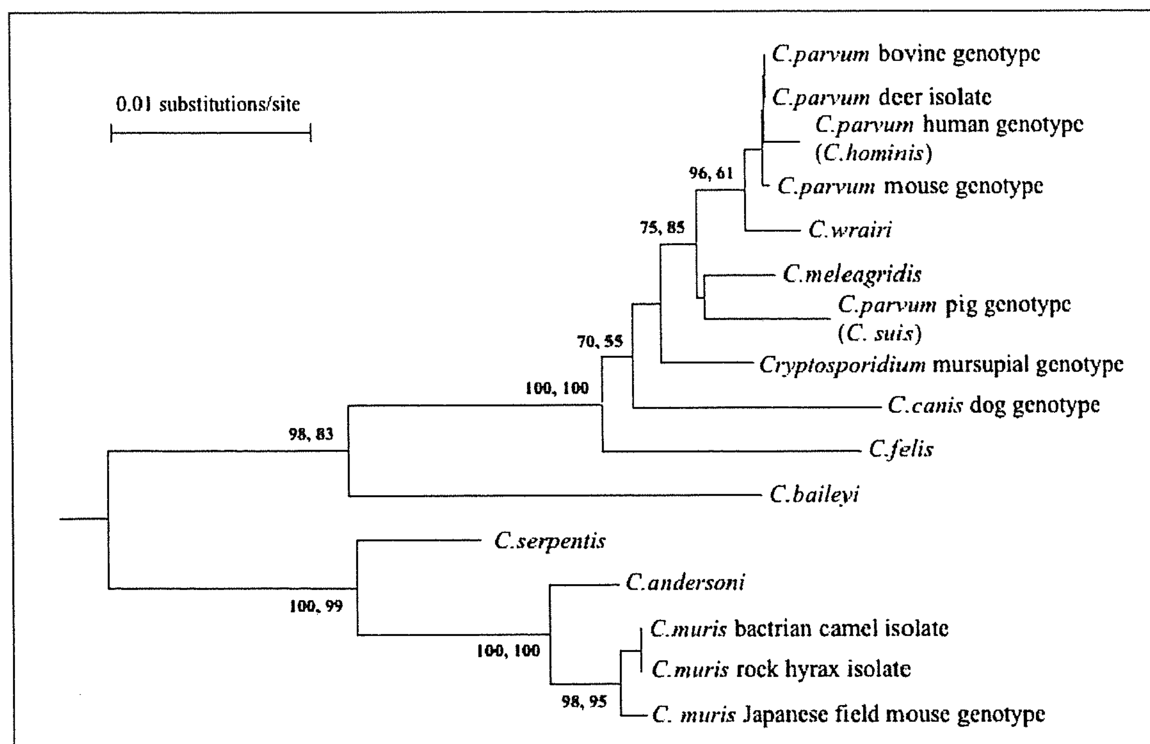
Oocysts used were purified by the sugar centrifugal flotation method [2]. DNA from each sample was extracted after five rounds of freezing and thawing of oocysts. Three sets of specific primers were used to amplify fragments of genes, namely, 18S ribosomal DNA (rDNA), the gene for heat shock protein 70 (HSP70) and the gene for *Cryptosporidium* oocyst wall protein (COWP) [7]. Subcloned product amplification by PCR were sequenced on an automated sequencer (ABI 310). By using the sequence data, we performed distance-based analysis using Kimura's distance formula and then we constructed a phylogenetic tree using MEGA. To assess the infectivity of the isolate, we inoculated purified oocysts orally into newborn calves, SCID mice or large Japanese field mice. The feces of each animal were collected, and the discharge of oocysts was monitored microscopically and genetically.

Results

We detected ovoid *Cryptosporidium* oocysts in feces from 6 of 113 cattle. The cattle discharged oocysts for the entire grazing period (143 days). Long patent periods were a characteristic of bovine *Cryptosporidium*. We also detected oocysts in feces from 2 of 28 calves that were born during the grazing. The average dimensions of these oocysts ranged from 7.4 by 5.1 to 7.6 by 5.9 μm, whereas those of *C. muris* RN66 and *C. parvum* HNJ-1 are 8.1 by 5.1 μm and 4.8 by 4.2 μm, respectively. Postmortem examination of a 3-year-old cow revealed *Cryptosporidium* at various stages of development on the epithelial cells of the abomasums [4]. The nucleotide sequence of 18S rDNA from the



isolate was identical to that from a bovine strain of *C. muris* (GenBank accession no. AF093496). Although only a sequence of 265 bp that was within the region we sequenced was registered for *C. andersoni* in GenBank (accession no. AJ275963), this sequence was homologous to that obtained from the new isolate. Partial sequences of genes for HSP70 and COWP from the isolate were coincident with published data for bovine isolates of *C. muris* (AF221542) and *C. andersoni* (AF266262). All of eight SCID mice discharged oocysts after oral inoculation of the isolate. The prepatent period for the isolate was 14 days in all mice, while that of *C. muris* RN66 was 6 days. The present isolate was infective to SCID mice, but it has been reported that *C. andersoni* did not infect SCID mice. Thus, the present isolate differed from published strains of *C. andersoni* and might be a novel type of *C. andersoni*. We refered to this strain as the *C. andersoni* strain of Kawatabi [6]. The similarity in terms of the nucleotide sequences of *C. andersoni* Kawatabi and of other strains of *C. muris* (GenBank accession nos. AF093497 and AF093498) was 98.81%. The sequence similarity between the *C. parvum* isolate from deer (GenBank accession no. AF093494) and the *C. parvum* isolate from marsupial (GenBank accession no. AF108860) was 98.89%. Nevertheless, the value for *C. parvum* (marsupial strain) and *C. wrairi* (GenBank accession no. AF115378), which are considered to be distinct species, was 98.93%. Thus, we could not conclude from the sequence similarity alone that the Kawatabi strain was distinct from *C. muris*. Further study may be needed for strict discrimination between *C. andersoni* and the Kawatabi strain.

Fecal samples were collected from 325 adult cattle in a slaughterhouse in Hokkaido, the northern island of Japan [3]. Five adult cattle were found to be positive for oocysts of *Cryptopsoridium* (1.5%). The oocysts were morphologically similar to those of *C. andersoni*. The partial sequence of the 18s rRNA gene of the isolate was 100% identical with that of the *C. andersoni* Kawatabi strain. SCID mice were infected after oral administration. Based on the morphology of the oocysts, the sequence of the 18S rRNA gene, and the infectivity to SCID mice, the isolate was concluded to be of the same type of *C. andersoni* Kawatabi strain that was isolated in Honshu, the main island of Japan.

Cryptosporidium muris-like oocysts were isolated from 2 of 25 large Japanese field mice, *Apodemus speciosus* captured in the farm where *C. andersoni* Kawatabi strain was isolated. Morphologically, these oocysts resembled those obtained from a *C. andersoni* Kawatabi isolate but were smaller in size than those from a *C. muris* isolate. Following oral inoculation of the oocysts into large Japanese field mice and SCID mice, developing stages were found in the stomach epithelium. The infectivity of the isolate to wild and laboratory mice was slightly different from that of *C. muris*. DNA sequences of the 18S ribosomal RNA (rRNA) gene of the isolate were not identical to those of any known *Cryptosporidium* spp.; however, phylogenetic analysis indicated that the isolate was a member of the *C. muris* cluster. Differences between the isolate and *C. muris* are not significant at this point; therefore, we proposed that this isolate is a novel genotype of *C. muris*, and denote it as *Cryptosporidium muris* Japanese field mouse genotype [1]. However, there remains a possibil-

ity that the isolate is a novel species. We need to reconsider the nomenclature for *Cryptosporidium* spp., especially for gastric type of the parasites.

Conclusions

1. *C. muris* type oocysts were isolated from beef cattle in the main island and northern island of Japan, and from large Japanese field mice in Japan.
2. *C. andersoni* Kawatabi type was denoted as a novel type of *C. andersoni* that parasitized in the abomasum of cattle and the stomach of SCID mice.
3. The isolate from the field mice parasitized to the stomach of the wild mice and SCID mice.
4. Phylogenetically, the isolate was a member of the *C. muris* cluster, and was denoted as *Cryptosporidium muris* Japanese field mouse genotype as a novel genotype.

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Vet Pathol 43:202–207 (2006)

Morphological and Immunohistochemical Features of *Cryptosporidium andersoni* in Cattle

K. Masuno, T. Yanai, A. Hirata, K. Yonemaru, H. Sakai, M. Satoh, T. Masegi, and Y. Nakai

Abstract. Light and electron microscopic features and immunohistochemical features of *Cryptosporidium andersoni* (*C. andersoni*) and host reaction in the mucosa were studied. Although the affected cattle demonstrated no apparent clinical signs, a severe infection of *C. andersoni* was observed in the abomasum. *C. andersoni* were round in shape, measured 6–8 µm in size and were mainly observed to be freely located in the gastric pits, being attached in occasional cases to the surface of the abomasum epithelium. Frequent inflammatory cells had infiltrated the lamina propria of the affected mucosa, and frequent mitotic figures were observed in epithelial cells at the dilated isthmus. To access the cell kinetics, the number of epithelial cells infected with *C. andersoni* were counted and compared with noninfected cattle. The number of gastric pit cells in infected cattle was significantly higher than that in the controls. The number of proliferative cells determined by the Ki-67 antigen in *C. andersoni* infected cattle was also significantly higher than that in the controls. Transmission electron microscopy and scanning electron microscopy revealed that the morphology of the *C. andersoni* organism was common to those of other *Cryptosporidium* spp. Immunohistochemically, several commercial antibodies against *Cryptosporidium* spp. showed positive reactions at the wall of these oocysts or parasitophorous vacuoles. This report is possibly the first to discuss the prominent hyperplasia of the abomasum mucosa, as well as morphologic features of *C. andersoni* in cattle.

Key words: Abomasum; cattle; cryptosporidium; hyperplasia; immunohistochemistry; ultrastructure.

Genus *Cryptosporidium* is an intracellular protozoan parasite and causes gastrointestinal disease in a wide variety of mammals and other vertebrate species worldwide.[1,9,11] *Cryptosporidium* spp. are transmitted by the ingestion of oocysts that are excreted in the feces of humans and animals.[9,14,17,22] In cattle, three subspecies of *Cryptosporidium* have been reported: *Cryptosporidium parvum*,[9,10,20] *Cryptosporidium felis*,[3] and *Cryptosporidium andersoni*, recently renamed from *Cryptosporidium muris*.[2,4,8,10,12,13,18–20]

Cryptosporidium resides on the apical surface of intestinal epithelial cells. It is viewed as a minimally invasive, mucosal pathogen, which causes infection at the microvillus border of the gastrointestinal epithelium. However, infection elicits a cell-mediated response following both primary and secondary infections.[9] Some *Cryptosporidium* spp. cause acute diarrhea in immunocompetent animals and chronic life-threatening disease in immunocompromised animals.[15,23]

Many studies have been conducted on morphology, immunohistochemistry and epidemiology for some species of *Cryptosporidium*, particularly *C. parvum*.[5,9] *C. andersoni* is a newly established member of *Cryptosporidium* spp., and there have been few reports on morphologic studies in cattle, including histopathological and ultrastructural features, as well as immunohistochemical reactivity. Furthermore, there has been no study on the histopathological alterations in the mucosa of host animals.

In the present study, we attempted to clarify the histopathological characteristics of host reactions in the mucosa, as well as morphological characteristics of *C. andersoni*, by using light and electron microscopy.

Eight Japanese black hair cattle with or without fecal excretion of *Cryptosporidium* spp. were examined. Four cattle (Nos. 1 and 3–5) were obtained from the farm of Tohoku University in Kawatabi, Miyagi, Japan. The other cattle (Nos. 2 and 6–8) were kept at dairy farms in Gifu Prefecture and were sent to Gifu University. In four cattle maintained at the farm of Tohoku University, oocysts of *Cryptosporidium.* spp. were collected from fecal samples using the sucrose flotation method and were identified as *C. andersoni* by sequencing the 18S ribosomal DNA, heat-shock protein 70 and oocyst wall protein genes.[18] Oocysts of *C. andersoni* were defecated for at least 14 months in cattle No. 1; 31 months in cattle Nos. 3 and 4; and 35 months in cattle No. 5 before euthanization, respectively (Table 1). The cattle were euthanatized, and complete necropsy was done. Organs and tissues, including the liver, spleen, kidneys, heart, lungs, rumen, reticulum, omasum, abomasum, ileum, cecum, colon, and adrenal glands, were collected and fixed in 10% buffered formalin and embedded in paraffin. For the abomasum, three samples were collected from the anterior, middle, and posterior parts. Specimens of abomasum from three Japanese black hair cattle with no lesions were used as a control (Nos. 6–8). Each of the sections was cut at

Table 1. Summary of the clinical data of cattle.

Cow No.	Age	Sex*	Origin	Period of Shedding Oocyst	Species of *Cryptosporidium*
1	4y2m	F	Miyagi	<14m	*Cryptosporidium andersoni*
2	6y	F	Gifu	ND	*Cryptosporidium* spp.
3	3y	F	Miyagi	31m	*Cryptosporidium andersoni*
4	3y	F	Miyagi	31m	*Cryptosporidium andersoni*
5	3y1m	F	Miyagi	35m	*Cryptosporidium andersoni*
6	9m	M	Gifu	ND	ND
7	5m	M	Gifu	ND	ND
8	10m	M	Gifu	ND	ND

* M = male; F = female; ND = not done.

a thickness of 4 μm, stained with hematoxylin and eosin (HE), and examined by light microscopy. In the three infected cattle (Nos. 3–5) and the three control cattle (Nos. 6–8), the number of cells was counted at 10 gastric pits per cow.

Samples of the abomasums from three cattle (Nos. 3–5) were prepared for transmission electron microscopy (TEM). The specimens were rinsed in phosphate buffer, postfixed with 1% buffered osmium tetroxide, dehydrated in ethanol and propylene oxide, and embedded in epoxy resin. Individual samples were sectioned at 1 μm and stained with toluidine blue. Areas where *C. andersoni* were present were selected by light microscopy, and thin-cut sections, mounted on copper grids, were stained with lead citrate/uranyl acetate and examined with TEM (Hitachi, Tokyo, Japan, H-8100). Samples of formalin-fixed abomasum from one cow (No. 3) were washed in phosphate buffer and directly examined with scanning electron microscopy (SEM) (Hitachi, Tokyo, Japan, S-3000N) under low-vacuum conditions (150 Pa).

Immunohistochemical stain was determined in four of the infected cattle (Nos. 1, 3–5) and three of the control cattle at the anterior part of the abomasum. The stain was performed on prepared slides using the avidin-biotin-horseradish peroxidase (ABC) method. The primary antibodies used were mice monoclonal antibodies against *Cryptosporidium parvum* oocyst (Chemicon, Temecula, CA), *Cryptosporidium parvum* (Argene, Varilhes, France), and *Cryptosporidium* (VMRD, Pullman, WA). For analysis of cell kinetics at the affected sites, immunohistochemistry for Ki-67 antigen was conducted, and examination was made by light microscopy. In the three infected cattle (Nos. 3–5) and three control cattle (Nos. 6–8), the total number of epithelial cells and the number of Ki-67-positive epithelial cells were counted at 10 gastric pits per cow, and the percentage of Ki-67-positive epithelial cells in the three infected cattle was compared with the three noninfected controls. The number of gastric pit cells and the incidence of Ki-67-positive cells in the affected cattle and noninfected control cattle were expressed as averages ± SD and compared using the Wilcoxon signed rank test. Differences between groups were considered significant if the probability values evinced under 0.01.

Grossly, no abnormalities were observed in any organs or tissues, including the gastrointestinal tract, in any case examined.

Histopathologically, no abnormalities were detected except in the abomasum. In the abomasum of all five positive cases, numerous weakly basophilic, round-to-oval organisms measuring 6–8 μm in size were observed on the apical surface of the epithelium in the gastric pits at the anterior and middle parts (Fig. 1). The length of the gastric pits was significantly extended, and the number of epithelial lining cells had increased in comparison with the noninfected control cattle (Fig. 2a, 3a). The average number of gastric pits cells for three *C. andersoni* infected cattle (291.9 ± 55.3 SD) was found to be significantly greater than that of the three control cattle (146.7 ± 29.2 SD). The level of significance was less than 0.01 in nonparametric testing. Many fragment nuclear epithelial cells were observed at the isthmus of the gastric pits (Fig. 1). In the lamina propria of the affected mucosa, mild-to-moderate diffuse infiltration of lymphoid cells,

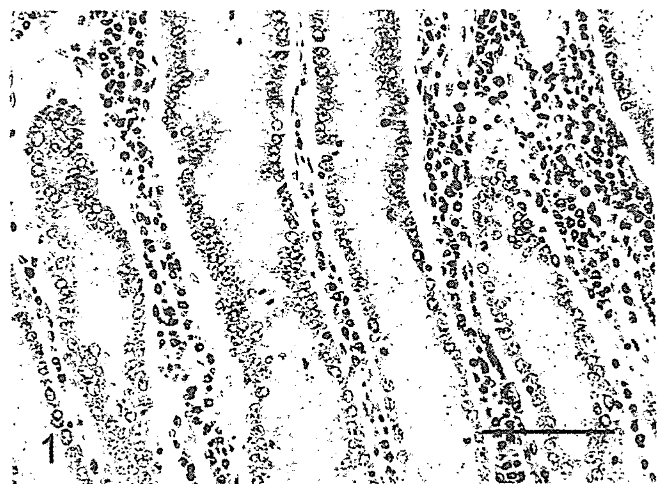


Fig. 1. Abomasum; cow No. 3. Numerous *C. andersoni* in gastric pits with diffuse infiltration of inflammatory cells in the lamina propria, and mitotic figures of epithelial cells. HE, paraffin section. Bar = 100 μm.

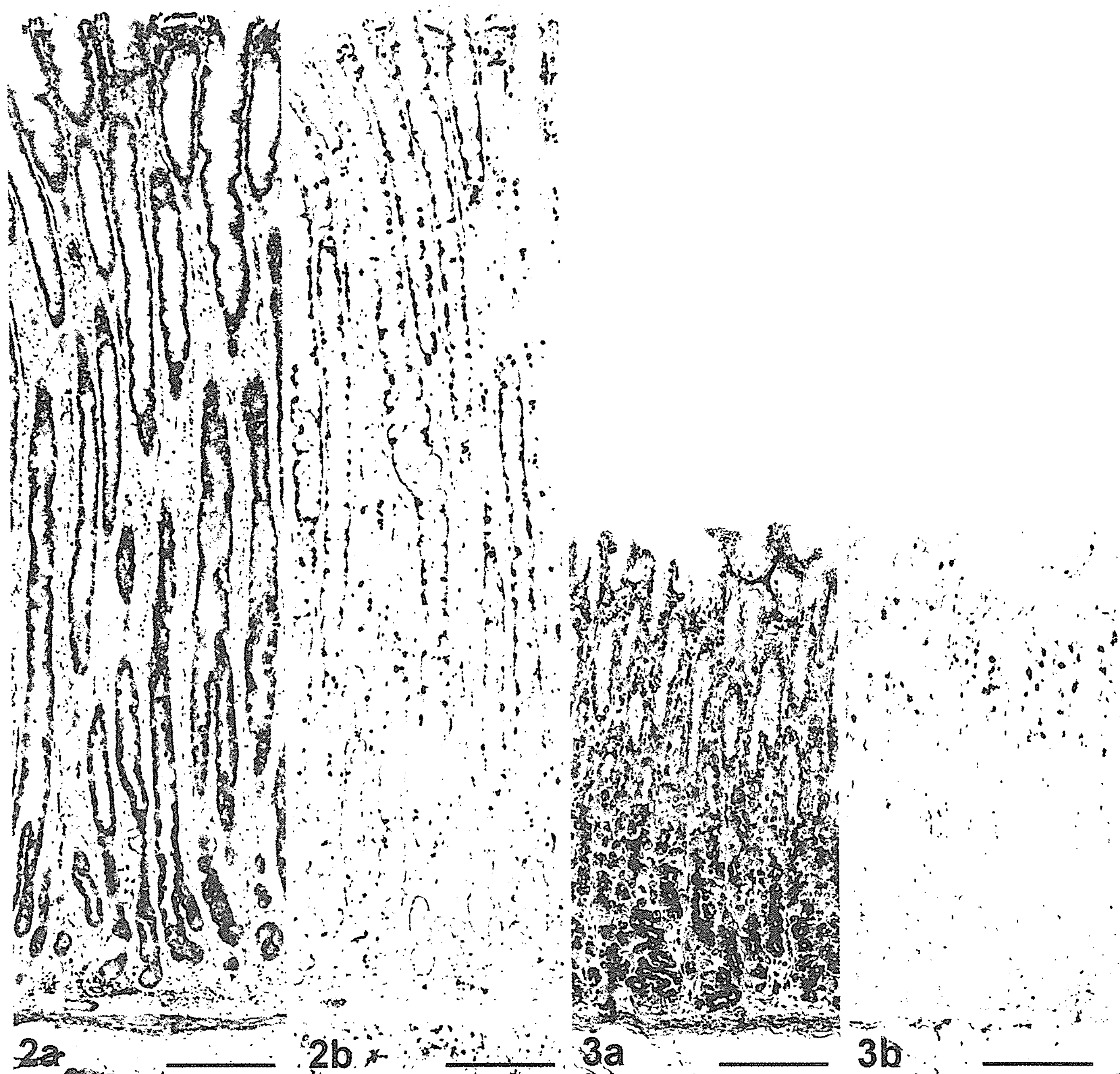


Fig. 2a. Abomasum; cow No. 5. The length of the gastric pits is greatly extended because of increase in the number of epithelial cells. HE, paraffin section. Bar = 200 µm. **Fig. 2b.** Abomasum; cow No. 5. Cells with brown nuclei in all active phases of the cell cycle. The length of the gastric pit is greatly extended due to increased numbers of gastric pit cells. Immunohistochemical stain for Ki-67 antigen, paraffin section. Bar = 200 µm.

Fig. 3a. Abomasum; cow No. 6. No lesions. HE, paraffin section. Bar = 200 µm. **Fig. 3b.** Abomasum; cow No. 6. No lesion. Immunohistochemical stain for Ki-67 antigen, paraffin section. Bar = 200 µm.

plasma cells, and eosinophils and mild edema were observed (Fig. 1). There were no lesions in the mucosal epithelium of the posterior part of the abomasum.

TEM revealed various life-cycle stages of *C. andersoni* on the epithelial surface of the gastric pits. Trophozoites enveloped the microvilli lining on the surface of the mucosal epithelium in the gastric pits, and were located within a parasitophorous vacuole. They exhibited well-developed feeder organelle and a rough endoplasmic reticulum in the cytoplasm (Fig. 4). Generally, schizonts contained eight merozoites within a parasitophorous vacuole (Fig. 5).

Under SEM, numerous round-to-spherical organisms consistent with *C. andersoni* were observed on the

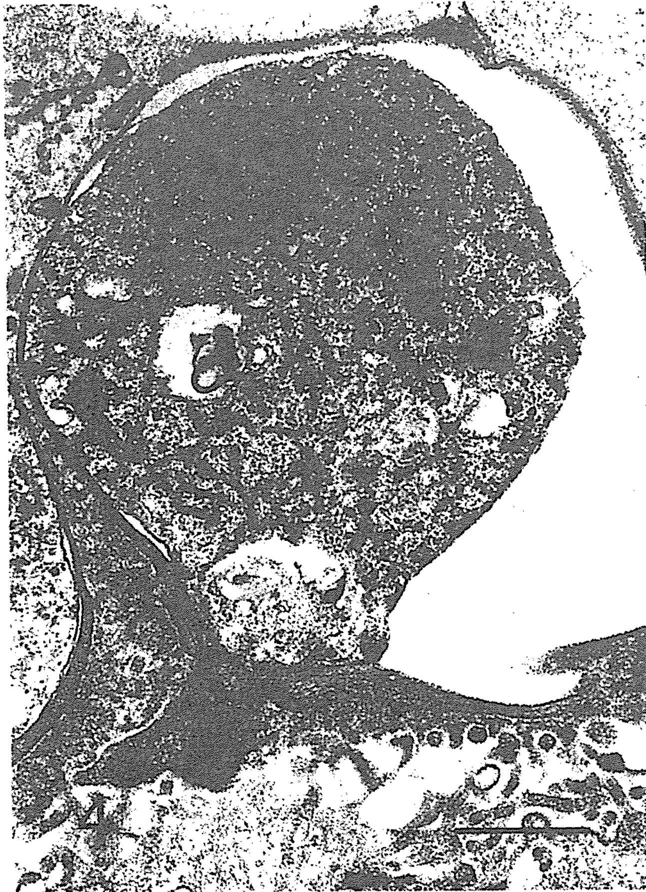
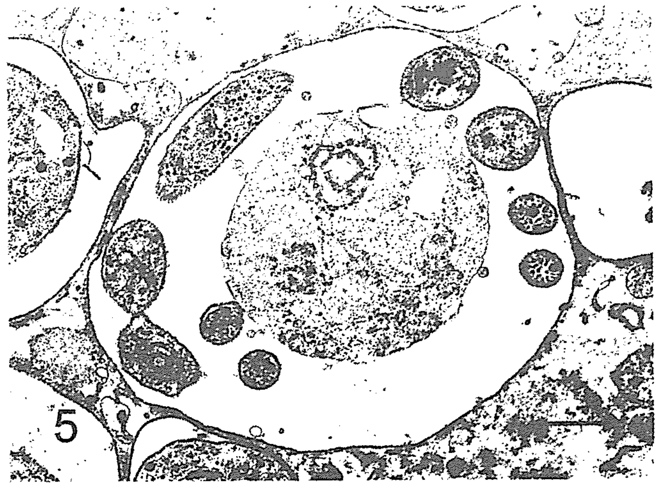
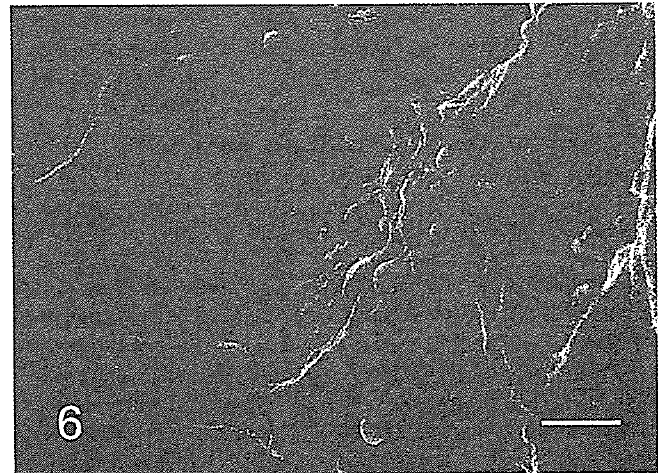


Fig. 4. *C. andersoni*; cow No. 3. Trophozoite attaching to the epithelial cells of gastric pits with large parasitophorous vacuole. TEM. Bar = 1 μm.





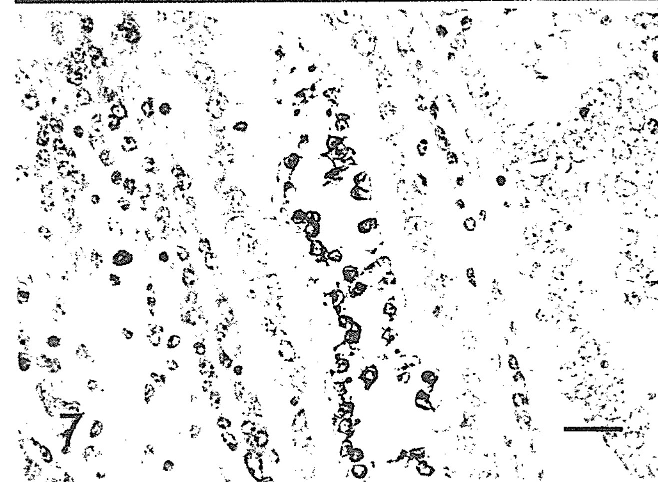


Fig. 5. *C. andersoni*; cow No. 3. A mature shizont with eight merozoites in the parasitophorous vacuole. TEM. Bar = 1 μm.

Fig. 6. Abomasum; cow No. 3. Large numbers of *C. andersoni* on the epithelial cells. SEM. Bar = 20 μm.

Fig. 7. Abomasum; cow No. 3. Only the wall of parasitophorous vacuoles and oocysts of *C. andersoni* positive for primary antibody. Immunohistochemical stain for *Cryptosporidium parvum* oocyst (Chemicon, Temecula, CA), paraffin section. Bar = 20 μm.

surface of the abomasum mucosal epithelium (Fig. 6). Occasionally, hatching organisms of *C. andersoni* and the remaining shells were observed.

Immunohistochemically, in the infected cattle that were examined (Nos. 1 and 3–5), most of the weakly basophilic, oval organisms in the gastric pits showed a positive reaction for primary antibodies against *Cryptosporidium parvum* oocyst (Chemicon, Temecula, CA), *Cryptosporidium parvum* (Argene, Varilhes, France) and *Cryptosporidium* (VMRD, Pullman, WA) (Fig. 7). Those organisms that were reactive to antibodies were larger in size than those observed with HE stain. The Ki-67 antigen–positive proliferation cells were clearly identifiable by their brown nuclei. A large number of epithelial cells showed positive reaction in the cervix portion of the gastric pits (Fig. 2b). The length of the cervix portion of the gastric pits was largely extended in comparison with the noninfected control cattle (Figs. 2b, 3b). The average number of Ki-67-positive index in the gastric pits in the three cattle with *C. andersoni* infection was 56.2% ± 11.1 SD, and the average number in the corresponding control cattle was 47.2% ± 16.9 SD. The average frequency of the proliferative cells in the gastric pits of infected cattle was significantly higher than that of

the controls. The level of significance was less than 0.01 in nonparametric testing.

HE sections revealed that *C. andersoni* infection was specifically limited to the mucosa of the abomasum in the present study. On the other hand, infection caused by other *Cryptosporidium* spp., such as *C. parvum,* showed up in the small intestine, particularly in the ileum. Unlike *C. parvum*, *C. andersoni* and *C. muris* were thought to have different infection sites. This is a characteristic feature of these species. Furthermore, on the same abomasum, there was a clear predisposition to infection of *C. andersoni*, which was located in the anterior and middle parts of the abomasum. These predisposed infection sites in the abomasum may be dependent on environmental factors, such as favored pH, ion balance, or the characteristics of the host cells, but detailed factors were not fully clarified.[4,6]

As with the in vitro examination of *C. parvum*, *C. andersoni*[4,6,9] showed increased nuclear fragmentation and enhanced apoptosis in the epithelial cells of the affected area in the present study. It was hypothesized that the cell division served as defense mechanisms or host response against attachment and invasion by *C. andersoni* organisms.

The length of the gastric pits was extended as a result of the accumulation of increased epithelial cells in the cervix portion, and the proliferation portion was not the principalis of the gastric pits, as it is in monkeys with *Cryptosporidium muris*-like infections.[7] The number of the gastric pit cells in cattle with *C. andersoni* had significantly increased ($P < 0.01$). This increase in the number of cells could conceivably correlate strongly with the larger frequency of Ki-67-positive cells. This result indicated that the extension of the isthmus of the gastric pits might be caused by the increasing number of cells as a consequence of the high frequency of cell divisions.

A moderate degree of infiltration of lymphocytes, plasmacytes, and eosinophils was found in the lamina propria in the affected abomasum. The severity of the inflammatory cell reaction in the lamina propria was more intense in infections caused by other *Cryptosporidium* spp., especially *Cryptosporidium parvum*, than in those caused by *C. andersoni*.[6,8,9,16,21] This relatively mild host reaction might relate to the long term colonic infection.

The ultrastructural features of *C. andersoni* were similar to those in other *Cryptosporidium* spp. for the most part,[7,9] except for the size of the parasitophorous vacuole. The size of the *C. andersoni* parasitophorous vacuole was larger than that in other *Cryptosporidium* spp. This may be one of the characteristic features of *C. andersoni* on TEM observation. SEM revealed numerous *C. andersoni* organisms with a spherical shape attached to the surface of the mucosal epithelium of the abomasum. We concluded that *C. andersoni* mainly infected the apical surface of the pits of the abomasum and then grew and propagated in the abomasum.[5]

Immunohistochemically, most *C. andersoni* organisms were reactive to all primary antibodies examined. However, *C. andersoni* with positive reactivity by immunohistochemistry were larger than those examined with HE stain. This discrepancy might be due to the presence of parasitophorous vacuole around the organisms. TEM observation showed that the size of the parasitophorous vacuole of *C. andersoni* was much larger than the size of the nucleus and cytoplasm, which might account for the discrepancy in size between the HE stain and immunohistochemical stain.

In conclusion, most histological and ultrastructural features of *C. andersoni* organisms and its mechanisms of attachment to epithelial cells might be common on the whole to those in other *Cryptosporidium* spp. However, the host response was different. Only in *C. andersoni* mucosal hyperplasia was observed.[9a] It is unknown whether the same phenomenon occurs in other animals. Further studies are needed to clarify the pathogenesis of *C. andersoni* in animals including humans.

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SHORT COMMUNICATION

Characterization of *Cryptosporidium canis* isolated in Japan

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Abstract Oocysts that morphologically resemble *Cryptosporidium canis* oocysts were isolated from a stray dog captured in the northeastern part of the main island of Japan. The DNA sequence of the 18S rRNA gene of the isolate showed high homology to the published sequence of *C. canis* that was isolated in USA by Fayer et al., *J Parasitol*, 87:1415–1422, (2001). The isolate phylogenetically belonged to the *C. canis* cluster; however, its DNA sequence showed two base substitutions. This suggests the genetic diversity of *C. canis*.

Introduction

Cryptosporidium oocysts were isolated from a dog with persistent diarrhea, and the 18S rRNA gene sequence was deposited as *Cryptosporidium parvum* dog genotype (Morgan et al. 1999; Xiao et al. 1999). Because the gene showed low homology, Fayer et al. (2001) proposed that it be classified as *Cryptosporidium canis*. However, it is still unclear whether the major species of *Cryptosporidium* isolated from dogs are *C. canis* or genetic diversity exists among these species.

Cryptosporidiosis accompanied by persistent diarrhea in dogs was first reported by Wilson et al. (1983), and it had been considered as a *C. parvum* dog genotype based on the nucleotide sequence of genes for the 18S rRNA, a 70-kD heat shock protein (HSP 70), and the oocyst wall protein (COWP) (Sulaiman et al. 2000; Xiao et al. 1999, 2000). Fayer et al. (2001) proposed that the new species should be classified as *C. canis* based on the following observations: (1) infectivity to cattle and no infectivity to mice, (2) the genetic distance of *C. canis* from *Cryptosporidium hominis* (previously considered to be the *C. parvum* human genotype or genotype 1) or *C. parvum* (previously known as the *C. parvum* bovine genotype or genotype 2) was greater than that of other genotypes, namely, *C. parvum*, *Cryptosporidium wrairi*, and *Cryptosporidium meleagridis*. *C. canis* is now considered as a valid species (Xiao et al. 2004).

In this study, *Cryptosporidium* oocysts that were isolated from a stray dog were characterized on the basis of morphological, biological, and genetical analyses.

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Materials and methods

Fecal samples were collected from 294 dogs and 31 cats that were captured or carried to an animal center in the northern part of Miyagi prefecture. Miyagi prefecture is located in the northeastern part of the main island of Japan. Oocysts were detected by using the sugar centrifugal flotation method (Satoh et al. 2003). In brief, 1 g of fecal sample was mixed with a sodium acetate-acetic acid-formalin (SAF) solution, centrifuged, and then the supernatant was removed. Next, the supernatant was mixed with a 56% sugar solution and centrifuged. The surface of this

mixture was observed under a microscope. The detected oocysts were collected and purified by the sugar centrifugal flotation method (Nakai et al. 2004). After five cycles of freezing and thawing the oocysts, DNA from the sample was extracted by using the MagExtractor Genome kit (Toyobo, Osaka, Japan). A primer set was used to amplify the complete sequence of the 18S rRNA gene (Xiao et al. 1999). As the reference strain, we used *C. parvum* HNJ-1 that was originally isolated from a Japanese woman (Masuda et al. 1991). This strain was previously classified as *C. parvum* bovine genotype or genotype 2, and it has recently been defined as a *C. parvum* (Satoh et al. 2005). In our laboratory, this strain was passaged in SCID mice.

The polymerase chain reaction (PCR)-amplified products were cloned by using the pT7Blue-2 Perfectly Blunt Cloning Kit (Novagen) according to the manufacturer's instructions. Three clones were sequenced by using an automated DNA sequencer (ABI 310; Applied Biosystems Japan, Tokyo, Japan). The sequence accuracy of the data was confirmed by bidirectional sequencing. To compare this sequence with the sequences of *C. parvum* genotypes registered in GenBank, we performed a distance-based analysis using Kimura's distance formula and constructed a phylogenetic tree using MEGA version 2.1 (Kumar et al. 2001). For phylogenetic analysis, a tree was constructed by using the neighbor-joining method on the basis of the arithmetic mean that was obtained from the aligned sequences of the 18S rRNA genes from various known genotypes of *C. parvum*.

To assess the infectivity of the isolate, two 4-week-old SCID mice were orally inoculated with 10^5 purified oocysts. The fecal sample of each mouse was collected and the amount of oocysts discharged was monitored microscopically for 28 days, as described previously (Satoh et al. 2003).

Results and discussion

In this study, although no oocysts were detected in any of the cats, we detected *Cryptosporidium* oocysts in 1 of the 294 dogs (0.3%). The dog that discharged the oocysts was captured from a mountainous area in a village, where agricultural farms were located and dairy and beef operations were carried out. The average size of the oocyst was $4.4\pm0.5\times3.2\pm0.4$ μm (length to width ratio: 1.34, $n=50$). We referred to these oocysts as the Hanayama isolate. The average size of these oocysts was smaller than that of the *C. parvum* HNJ-1 strain (average size: $4.8\pm0.4\times4.2\pm0.5$ μm, $p<0.05$) and *C. canis* (average size: 4.95×4.71 μm) (Fayer et al. 2001). The length to width ratio of the Hanayama isolate was 1.34 but that of *C. parvum* and *C. canis* was 1.04–1.06. This indicated that the isolate had an elliptical shape. This suggested the existence of morphological diversity with regard to the shape and size of oocysts in *C. canis*.

PCR was performed to amplify the 1741 bp sequence of the 18S rRNA gene of the Hanayama isolate; the determined sequence has been deposited in GenBank (accession no. AB210854). Two base pair substitutions at positions 1,684 and 1,685 were observed on comparison with the *C. parvum* dog genotype that is registered in GenBank (accession no. AF112576). Because Fayer et al. (2001) have proposed this registered genotype as a new species *C. canis*, we also considered the *C. parvum* dog genotype as *C. canis*. The constructed phylogenetic tree revealed that the Hanayama isolate belongs to the cluster of *C. canis* (Fig. 1). PCR was also performed using primer sets targeting HSP 70 and COWP of *C. parvum* that were used in a previous study (Satoh et al. 2005); however, no PCR fragments were obtained (data not shown). Morgan et al. (2000) have reported the heterogeneity of the HSP 70

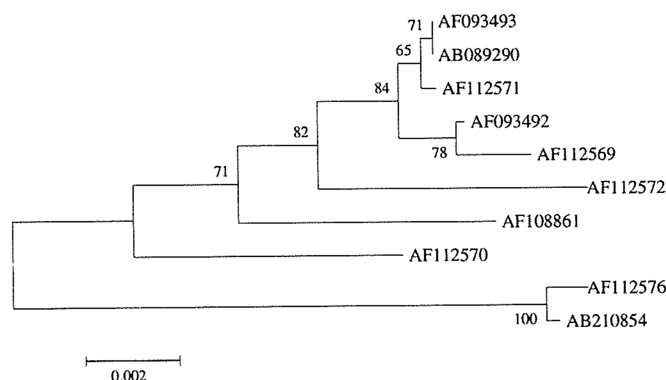

Fig. 1 The evolutionary relationship of our isolate in this study inferred by NJ analysis of Tamura-Nei distances calculated form pairwise comparisons of gene sequences for 18S rRNA using MEGA version 2.1. Percentage of bootstrap support from 1,000 replicate samples is indicated at each node. Each accession number indicates the 18S rRNA sequence registered in GenBank: AB210854, Hanayama isolate; AF093492, *C. hominis*; AF093493, *C. parvum*; AB089290, *C. parvum* HNJ-1 strain; AF112576, *C. canis*; and AF112571, AF112569, AF112572, AF108861, and AF112570 indicate mouse genotype, rhesus monkey genotype, ferret genotype, pig genotype, and kangaroo genotype of *C. parvum*, respectively

gene among the two dog genotypes isolated in USA and Australia, respectively. They observed partial 18S rRNA gene sequences of both isolates (nucleotide position: 1 to 713, corresponding to our data), and reported that these were identical. Abe et al. (2002a,b) isolated canine *Cryptosporidium* from a western region of Japan at a distance of approximately 800 km from Miyagi prefecture, where our investigations were carried out. The partial 18S rRNA gene sequence of this isolated canine *Cryptosporidium* was identical to that of *C. canis* (nucleotide position 445 to 734, corresponding to our data). Hanayama isolate and Fayer's isolate were identical to the species isolated in USA and Australia with regard to the positions from 1 to 713 and to Abe's isolate with regard to the positions from 445 to 734. To distinguish the isolates of *C. canis*, positions 1,684 and 1,685 were considered as the candidate sequence for DNA analysis. Xiao et al. (2004) observed that the genetic distance (0.38% in 18S rRNA) between the marsupial genotype from Australia and the opossum genotype from North America was small; this small genetic distance was attributed to the geographical distribution that had resulted from the continental drift. Widmer et al. (1998) demonstrated genetic diversity in the gene for β-tubulin among the same genotype of *C. parvum*, suggesting the population structure within the same genotype. Furthermore, we demonstrated that the *C. parvum* HNJ-1 strain has a heterogenous structure when compared to the other *C. parvum* strain (Satoh et al. 2005). Thus, the genetic difference (0.11%) in the 18S rRNA gene between the geographically separated Hanayama isolate and *C. canis* isolated in USA suggests the intraspecific diversities of *C. canis*. To clarify the extent of intraspecific diversity due to geographical distribution, further survey and molecular analyses of *C. canis* from different areas and countries must be conducted.

In this study, none of the SCID mice discharged oocysts for 28 days after the oral inoculation of the isolate. In this experiment, we used fresh oocysts isolated from a dog, and a considerably high dose of oocyst (10^5) was used for inoculation. Therefore, the Hanayama isolate may have low or no infectivity to SCID mice.

The characteristics of our isolate with regard to its infectivity in the laboratory mice was consistent with that of *C. canis*, as described previously; however, the two differed in terms of their morphological characteristics.

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Cyclospora and *Isospora* in Diarrheic Patients from the Philippines

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ABSTRACT

In recent years, *Cyclospora cayetanensis* and *Isospora belli* have been recognized as causative organisms of chronic diarrhea. This study was undertaken to determine the prevalence of enteric protozoa among diarrhea patients in the Philippines. From the collection of 3456 stool samples examined, only one sample each was found positive for oocysts of *Cyclospora cayetanensis* and *Isospora belli*. Identification was based on autofluorescence of the oocyts with a 365nm UV excitation filter. Both samples were obtained from male patients (18 and 73 years old respectively) living in Iloilo in the western islands of Visayas. Both patients obtained their drinking water from deep wells. The identification of these 2 emerging pathogens, which are easily overlooked by less-trained technical staff, highlights the increased technical capability in the Philippines. This capability will be further developed due to the greater awareness of the presence of such pathogens in the local setting.

Cyclospora, belonging to Family Eimeridae, Subphylum Apicomplexa, and *Isospora,* a coccidian parasite, are taxonomically related to *Cryptosporidium.* Like the latter, both are implicated as etiologic agents of diarrhea in immunocompromised persons, particularly AIDS patients. *Cyclospora,* first observed in humans in Papua New Guinea (1), has been isolated from humans worldwide with increasing frequency since 1985 (2). *Isospora belli* was first described in 1915 (2) and is the only species of *Isospora* known to infect humans. It has been reported in tropical areas of South America and Southeast Asia (3), and has also been associated with diarrhea outbreaks in mental wards and day care centers (2).

Previous reports have documented the presence of *Isospora* and *Cyclospora* in the Philippines. *I. belli* was detected in 5 out of 103 United States naval personnel and their families upon returning to the US from the Philippines after World War II (4). Faust *et. al* (5) also reported that there were American soldiers who were diagnosed with isosporiasis in the Philippines during World War I. Jueco *et al* (6) made the first report on the detection of *Isospora* from diarrhea cases among locals in the Philippines. A case of traveler's diarrhea caused by *C. cayetenensis* acquired in the Philippines was reported by Ohnishi *et al* (7). The patient stayed in the country for 5 days in 2001, and upon his return to Japan, had persistent diarrhea and weight loss.